Characterization of a Fibroblast Specific Cre-Recombinase and Its Potential Utilization in the Study of Tumor Progression

A Senior Honors Thesis

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Abstract

Mammary glands, by the very nature of their post-natal development require dynamic cell interactions and high levels of cellular organization post-partum. The gland can essentially be separated into two main compartments—the epithelial tissue and the supporting stromal fat pad. Since a majority of breast cancers are of epithelial origin and rapid proliferation and apoptosis are typically associated with these cells, most cancer research has primarily focused on understanding the epithelial-component of tumorigenesis and tumor progression. Recently, however, there has been increased interest in how the stroma communicates with the epithelia and how the tumor microenvironment might affect the behavior of the tumor itself. Genetic manipulation of the stroma, and more specifically the fibroblasts, has remained difficult until the discovery of the fibroblast-specific protein 1 (FSP1) and the elucidation of the promoter controlling its expression. Using the FSP1 promoter, a fibroblast-specific cre-recombinase was created to enable genetic manipulation of the stroma *in vivo* in order to study the importance of the microenvironment on tumor initiation and progression.

Introduction

Mammary glands, by the very nature of their post-natal development require dynamic cell interactions and high levels of cellular organization post-partum. The gland can essentially be separated into two main compartments-the epithelial tissue and the supporting stromal fat pad. The component involved in the production and secretion of milk is the epithelial tissue. It is

comprised of sac-like structures and lobules which are connected through a tree-like network of ducts responsible for funneling the milk to the nipple. The stroma makes up the rest of the gland and consists of the adipocytes, fibroblasts, endothelial cells, macrophage, lymphocytes, B cells, T cells, nerve cells, and extracellular matrix surrounding and supporting the epithelial cells (Mueller and Rusenig, 2004)

During puberty, cells in the terminal end buds rapidly proliferate and penetrate into the fat pad allowing the ductal tree to branch and elongate through the stroma from the main collecting ducts at the nipple. These end buds disappear after duct elongation is complete. With the onset of pregnancy the ducts undergo lateral branching, which ultimately terminates in the formation of acini structures, also called alveoli, for producing milk. Each acinus consists of a single layer of highly polarized cells that form a sac-like structure containing the hollow lumen on the interior. The epithelial cells are histologically distinct by the way they form rings or tubes through tight cell-cell junctions as well as their relatively large, cuboidal shape. On the exterior, the epithelial cell layer is surrounded by a thin layer of myoepithelial cells which contain smooth muscle actin and contract to secrete the milk. The basal lamina, or basement membrane, separates these two cell layers from the surrounding stroma. Late in pregnancy, nearly all of the stroma volume is replaced by the expanded ductal tree and alveoli, which begin to lactate after birth. After weaning, the process of involution begins and the alveoli regress as many of the epithelial cells undergo programmed cell death, otherwise known as apoptosis (Dunbar and Wysolmerski, 2001). In the mouse, a small number of these alveoli structures develop and then regress within the mammary gland every five days as a result of hormone production during its estrous cycle (Fata et al., 2001).

With these frequent episodes of proliferation and apoptosis, in addition to large scale changes associated with pregnancy, it is easy to imagine the potential danger for developing cancer should these cellular processes become deregulated. Since a majority of breast cancers are of epithelial origin and rapid proliferation and apoptosis are typically associated with these cells, most cancer research has primarily focused on understanding the epithelial-component of tumorigenesis and tumor progression. Recently, however, there has been increased interest in how the stroma communicates with the epithelia and how the tumor microenvironment might affect the behavior of the tumor itself.

Studies elucidating just how important the stroma is in proper formation of the mammary gland played an important role in laying the foundation for this area of research. It has been suggested that the response of the mammary gland to estrogen is mediated by the stroma and not the epithelia (Cunha et al., 1997). The transplantation of mouse mammary epithelia into the submandibular salivary gland stroma results in what resembles a normal salivary gland; however, the epithelia still secrete the milk protein α-lactalbumin. Thererfore, the stroma must have a very significant role in the processes that control the morphological development of the epithelial network of ducts but has no affect on the cytodifferentiation of the epithelial cells themselves. The extracellular matrix could be part of the reason for this remarkable result. Epithelial branching in the salivary gland requires the extracellular matrix protein fibronectin which is secreted by fibroblasts (Sakai et al, 2003).

The extracellular matrix consists of non-living macromolecules in the extracellular space such as fibrous and non-fibrous collagens, glycoproteins, and proteoglycans (Silberstein, 2001). All tissues are composed of cells and an extracellular matrix (ECM). The components of the ECM are synthesized and secreted by various types of cells including epithelial cells, smooth

muscle cells, and fibroblasts. Matrix metalloproteinases (MMPs), one type of the macromolecules comprising the ECM, have been linked to the loss of integrity of the microenvironment that can lead to tumors (Bissell & LaBarge, 2005; Sternlicht et al, 1999). Genetic equivalence is one of the main arguments for the significance of the microenvironment in tumorigenesis. Nuclei from malignant cancer cells have been placed in enucleated oocytes and eventually used to produce chimeric mice. The majority of tissues developed normally in these mice because the normal microenvironment was able to control the malignant nature of the cells (Bissell & LaBarge, 2005; Hochedlinger et al., 2004). This suggests that some sort of activated microenvironment is necessary for the initiation and progression of cancer.

A recent study hoping to reveal some of the genetic alterations that might lead to such an activated state analyzed genomic deletion of primary tumor sites from patients with invasive breast cancer and determined that there are nearly two fold more targets for genetic alterations in the stromal cells compared to the neighboring epithelial tumor cells (Fukino et al., 2004). Among these targets are the tumor suppressor genes *p53* and *PTEN*, two of the more frequently mutated and/or delete genes in sporadic cancers. The stromal alterations in most invasive breast carcinomas are marked by an increase in fibroblasts and a modified, collagenized ECM. These carcinoma-associated fibroblasts (CAFs) are reported to express greater amounts of extracellular matrix molecules, various molecules that modulate the ECM, and many growth factors relative to fibroblasts found in normal mammary glands and have a decreased ability to suppress proliferation of pre-carcinoma epithelial cells (Sadlonova et al., 2005; Kunz-Schughart, 2002). Most ECM macromolecules were thought to serve a purely structural role in aiding cell adhesion and migration by providing a framework to act upon; however, when these molecules are cleaved by integrin-receptors, some have cryptic functions not expressed by their full length

counterparts such as activating signal transduction pathways similar to those activated by cytokines which have been recorded in the initiation and progression of cancer (Schor and Schor, 2001; Juliano, 1996). The upregulation of matrix metalloproteinases 1 and 9 (MMP1 and MMP9) which activate cell-surface and ECM-bound growth factors have been shown to correlate with malignancy, but this correlation was only found to be in the presence of stromal fibroblasts (Mueller et al., 2004; Borchers et al., 1997).

Because fibroblast are known to lay down much of the scaffolding of the mammary gland including the basement membrane, along with their well-documented increase in secretion of many growth factors and extracellular molecules, the specific study of how genetic alterations in these cells affect tumorigenesis and tumor progression is a logical place to begin. Fibroblasts are non-vascular, non-epithelial, and non-inflammatory cells. They are the principal cellular component of the connective tissue and are largely responsible for its synthesis (Kalluri and Zeisberg, 2006). Fibroblasts are ubiquitous and highly heterogeneous; they are as diverse as the locations in which they are found. Fibroblasts are elongated cells which lack markers that indicate other cell lineages. There are markers that indicate fibroblastic phenotype; however, none of them are exclusive to and expressed in all fibroblasts. For example, vimentin is expressed in a wide variety of fibroblast, but it is also found in endothelial cells, myoepithelial cells, and neurons. This poses quite a problem when attempting to study fibroblasts *in vivo*. Of the known markers, the most specific marker appears to be the fibroblast-specific protein 1 (FSP1).

The results of the genomic analysis performed by Fukino et al. meant that there was a need for the ability to conditionally delete p53 and PTEN specifically in the stroma in order to establish what roles these two tumor suppressors might play in tumorigenesis and tumor

progression. The answer to that demand and the subject of this thesis is a fibroblast specific Cre recombinase fusing the S400a4 promoter with the coding sequence of the P1 bacteriophage Cre recombinase.

Cre recombinase of the P1 bacteriophage is a member of the integrase family of site-specific recombinases. Cre recombinase catalyzes recombination between two recognition sites called loxP sites (Nagy, 2000; Hamilton and Abremski, 1984). The loxP site consists of 34 bp which would randomly occur only once in ever 10^{18} bp. Considering that the mammalian genome is only 3 x 10^9 bp, it is very unlikely that the sequence would appear outside the phage genome, making it quite useful in mammalian genetics. The consensus sequence consists of two 13 bp palindromic sequences flanking and 8 bp spacer. The spacer is asymmetric and thus confers an orientation to the loxP site. One molecule of Cre recombinase recognizes each of the palindromic segments of the loxP site. The recombinase molecules come together to form an active tetramer which recombines the DNA between the two loxP sites at the 8 bp spacer. The recombination event can be one of inversion, deletion, or insertion depending upon the orientation and location of the loxP sites and requires no other co-factors or sequences to accomplish the recombination (Nagy, 2000; Voziyanov et al., 1999).

Material and Methods

Generation of *FSP-cre* transgenic mice.

The transgene vector for Fsp1-Cre-BGH was created by inserting the BGH poly-A signal and the multiple cloning site of pcDNA3 (Invitrogen, Carlsbad, CA) into a modified pBluescript II plasmid backbone (Stratagene, La Jolla, CA). The 3.1 kb *Fsp1* gene promoter (*S100a4*) (Okada et al., 1995; Okada et al., 1998) extending 1.9 kb upstream of exon 1 to the end of intron 1 (1.2

kb) was long-range PCR amplified (Long Template System, Roche, Indianapolis) from C57BL/6NTac genomic DNA and cloned into the multiple cloning site of the vector. The Cre recombinase ORF from pMC-Cre (Gu et al., 1993) was PCR amplified and inserted between the *Fsp1* promoter and the BGH poly-A signal. Cloning was completed by standard protocols and all fragments were verified by DNA sequencing. All microinjection constructs were injected into pronuclear stage FVB/N mouse embryos, as previously described (Overbeek et al., 1991; Taketo et al., 1991), after the Fsp1-Cre-BGH fragment was excised by *Pac* I (New England Biolabs, Beverly, MA) digestion and gel purified. The same construct was used to obtain the 2nd generation animals, with the injections done at a later time.

Southern Analysis

3 μg of phenol/chloroform extracted DNA was digested in a 25μL volume using EcoR1 endonuclease (New England Biolabs; 10:1) and separated on a 0.8% agarose gel. The DNA fragments were denatured in the gel for 30min in a 0.5N sodium hydroxide/1.5mM sodium chloride solution and then transferred overnight to a Hybond-N+ membrane (Amersham) by the capillary method using the same solution. The probe was generated by a Pac1/ Apa1 double digest of the plasmid above. The 4.3kb product, which contained the entire FSP-cre construct (minus the BGH region), was gel purified, extracted, quantified and labeled with P^{32} using the Rediprime system (Amersham). The probe was hybridized to the membrane overnight and washed 2 times 15min with each a high (250mM sodium phosphate/1mM EDTA 2% SDS) and low (50mM sodium phosphate/1mM EDTA 1% SDS) stringency solutions.

Genotyping of Transgenic Mice

DNA was isolated from mouse tail tips and genotyped by PCR analysis using the following primer sets: ***FSP-cre***, forward (ATGCTTCTGTCCGTTTGCCG), reverse (CAATGCGATG-CAATTTCCTC)-1082bp and ***Rosa26^{LoxP}***, common (AAAGTCGCTCTGAGTTGTTAT), ***wild-type*** (GCGGGAGAAATGGATAT)-550bp, ***transgene*** (GCGAAGAGTTTGTCCTCAACC)-260bp.

Tissue Processing and X-gal Staining

Tissue was excised and portions were processed for *in situ* X-gal staining. Tissue was fixed directly in paraformaldehyde (2% PFA/0.2% glutaraldehyde in a 100 mM sodium phosphate buffer, pH=7.4) for 2 to 2.5 hours at 4^{o}C, washed for 10 min twice in phosphate-buffered saline (PBS) and then stained in a 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal) solution [4 mM potassium ferricyanide (Sigma), 4mM potassium ferrocyanide (Sigma), 2 mM magnesium chloride (Sigma), 0.2% IGEPAL CA-630 (NP-40 substitute, Sigma), 0.1% sodium deoxycholic acid (Calbiochem) and 1 mg/mL X-gal (Gold Bio Technology, St. Louis, MO) in PBS for 18 hours at RT protected from light. X-gal-stained tissue was washed for 10 min twice with PBS and post-fixed in 10% neutral-buffered formalin (Richard Allen) for 48 hrs at RT. Samples were then paraffin embedded, cut into 5 μm sections and counter-stained with nuclear fast red (NFR) and H&E.

Immunohistochemistry

Primary and secondary antibodies were diluted in DAKO diluent (DAKO) and applied in the following pairs: guinea pig **anti-Cytokeratin** 8/18 (1:250, RDI-PROGP11; Research

Diagnostics) with biotinylated donkey anti-guinea pig (1:500, Jackson Immunochemicals); and rabbit **anti-α-SMA** (1:100, BD Biosciences) with biotinylated sheep anti-rabbit (1:500, Jackson Immunochemicals) 5 μm sections were deparaffinized as follows. The slides were heated in an oven at 60˚C for 10 minutes, then placed in xylene substitute twice for 5 minutes each. They were rehydrated using two rinses in 100% ethanol for 3 minutes, soaked in 95% ethanol for 3 minutes, and 70% ethanol for 3 additional minutes. The slides were rinsed in water for 2 minutes. Endogenous peroxidases were quenched with 3% hydrogen peroxide for 3 minutes and rinsed in PBS for 5 minutes. Sections were blocked with M.O.M. blocking reagent (Vector Labs) for 30min, rinsed, incubated with primary antibody for 30min, rinsed, incubated with a biotinylated secondary antibody for 15 minutes and rinsed. Following the biotinylated secondary antibody use, the Vectastain ABC reagent (Vector Labs) was applied for 15 minutes and rinsed. Sections were incubated with the peroxidase substrate (DAB from Vector Labs) for until the desired staining was achieved. The slides were rinsed in water for 5 minutes and dipped in Mayer's Hematoxylin for 2 seconds to counterstain the tissue. Sections were then rinsed in water for 5 minutes and dehydrated as follows: 2 times in 95% ethanol for 10 seconds each, 2 times in 100% ethanol for 10 seconds each, 2 times in xylene substitute, and once in xylene. The slides were then cover slipped using xylene-based mounting medium (Richard-Allan Scientific).

Images

Photographs of histological sections were taken with an Axio digital camera (Zeiss) mounted on the Axioskop microscope (Zeiss). Whole mount photographs were taken with a Coolpix 5700 digital camera (Nikon). Image files were processed using Photoshop 7.0 (Adobe) or AxioVision 4.3 software (Zeiss).

Transplantation of Mouse Mammary Glands

All mice were 8 weeks old. Cervical dislocation was used to euthanize the donor mouse which was then sterilized by emersion in 70% ethanol. The mammary gland from either the fourth, fifth, ninth, or tenth position was removed. The mammary gland was cut in half during the removal of the lymph node, and then placed in sterile saline. The recipient mouse was sedated and anesthetized using isoflurane (Abbott Laboratories). Hair was removed from the surgical area located on the interscapular region. The anesthetized mouse was moved into a sterile hood where the surgical area was sterilized using a Nolvastan scrub followed by 70% ethanol. A subdermal pocket was made using curved scissors. Application of a wound clip with an automatic feeding Michelle's wound clip applicator followed the insertion of the donor gland into the pocket. All dissection and operating tools were autoclaved and kept sterile through the procedure using 70% ethanol.

Results

Through a series of fortunate events during the generation of the *FSP-cre* mice, two different transgenic alleles were created. The first generation allele is the transgene expected to result from the recombination used to produce the *FSP-cre*. The second generation is the combination of this transgene and a fibroblast specific transactivator. The fortunate aspect is that the second generation *FSP-cre* is more specific than the first.

Southern analysis of the two fibroblast specific Cre recombinases easily distinguishes between the two. After a partial digest with the EcoRI restriction enzyme, the endogenous FSP1 gene appears as a very large segment of DNA approximately 10-11 kb in length. Because the first generation transgene contains a single internal EcoRI restriction site, the appearance of two

bands other than the endogenous FSP1 reveals that a single copy of the FSP-cre transgene is present in the genome. The shear size of the fragments in the second generation FSP-cre prevent knowing the exact number of copies inserted.

The visualization of *FSP-cre* expression requires the use of a conditional reporter. For the purposes of the study, the $Rosa26^{LoxP}$ (Rosalox) transgene was selected. This is a modification of the gene trap strain ROSA βgeo 26 in which a neo expression cassette has been inserted into the ROSA26 locus. This neo cassette consists of a triple polyadenylation sequence added to the 3' end of the coding region which is flanked by loxP sites and followed by a functional *lacZ* gene and another polyadenylation sequence. In the absence of Cre recombinase, the *lacZ* gene will remain silent due to the presence of the triple polyadenylation sequence which prevents readthrough. After the Cre recombinase-mediated excision of this silencer, the *lacZ* gene will be transcribed, translated, and used to visualize the expression of the *FSP-cre*.

In situ X-gal staining of FSP-cre embryos at E8.5 shows similar expression in the tails of both generations of the *FSP-cre* transgene. During mouse development, somitogenesis begins on embryonic day eight (E8) with the epithelialization and further differentiation of mesenchymal cells as they migrate and separate from the posterior presomitic mesoderm. The generation of the somites requires constant recruitment of cells to the paraxial mesoderm (Rossant and Tam, 2002). It is quite possible that fibroblastic characteristics are necessary for this process of mesenchymal migration, and thus the presence of the reporter. The formation of the thymus begins on the twelfth day of development (E12) in the endoderm of the third and fourth pharyngeal pouches and the neighboring mesenchyme. At this time, the thymic primordium buds into the mesenchyme and becomes surrounded by fibroblasts (Maronpot, 1999). The expression of the rosalox reporter in the craniofacial region and around developing bone tissue is

also noteworthy as *S100a4 (FSP1)* is a negative regulator of mineralization of bone tissue and osteoblast differentiation (Duarte et al., 2003).

In the adult mammary gland, FSP-cre expression is not exclusive to the fibroblasts, but it is exclusive to the stroma. Immunohistochemical staining verifies that epithelial cells positive for cytokeratin 8/18 are surrounded by cells expressing the reporter, but they themselves are not. All LacZ positive cells appear outside the thin ring of myoepithelial cells which form the outer boundary of the epithelial ducts. The other parts of the stroma that appear to express the *FSP-cre* are endothelial cells and pericytes. One key difference in the specificity of the two generations of *FSP-cre* is the positive staining of the lymph node in the first generation.

As stated before, fibroblasts are ubiquitous, and thus the characterization of *FSP-cre* expression must involve the entire mouse. In the adult mouse, the main organs to take note of are the brain, pituitary gland, heart, spleen, thymus, and lungs. The expression throughout the brain differs between the two FSP-cre recombinase constructs; however, most parts of the brain are affected in some way by both transgenes. The polygonal cells of the pars intermedia are responsible for the staining observed in the pituitary gland. The vasculature, as well as the fibroblasts, in the heart of the first generation *FSP-cre* mice stain positive for β-galactosidase expression; the second generation *FSP-cre* only affects the connective tissue around the heart. One of the most pronounced differences in expression between the two is in the spleen. When a whole mount of the spleen from the first generation is X-gal stained *in situ*, the entire organ appears almost black. A possible cause might be non-specific expression in a migratory inflammation and immune cell such as those which are prolific in the thymus considering that the spleen is a location for differentiation and maturation in hematopoesis. The spleen of the second generation is largely normal but with a sparse amount of Cre-positive cells of

undetermined lineage. The medulla of the thymus for the second generation FSP-cre and the cortex for both generations are free of *lacZ* staining. The medulla of the first generation, however, has a significant amount of spotted staining. The epithelial ducts of the salivary glands of both transgenic *FSP-cre* mice and the wild type express β-galactosidase, but these are epithelial cells that normally secrete digestive enzymes which break down sugars and should not be considered non-specific expression of the *FSP-cre* (For a complete overview of the localation of positive LacZ staining, refer to Table 1).

Genetic alterations in mammary epithelia has been done for years with little concern of affecting other aspects of the study because promoters like the mouse mammary tumor virus (MMTV) and whey acidic protein (WAP) promoters are specific for those highly differentiated cells. This is not the case for fibroblasts. The ubiquitous nature of fibroblasts that results in the expression of the *FSP-cre* throughout the body is a concern when attempting to study cancer. For example, the expression of *FSP-cre* in the pars intermedia of the pituitary gland poses quite a problem. Any changes produced in the pituitary could cause imbalances in hormone levels that could affect the rate of tumor progression apart from the genetic activation of the microenvironment surrounding the tumor. Another problem discovered through experimental trial is that test subjects can die from other complications before a mammary tumor actually forms. In response to this concern, the task of transplantation has been undertaken. By relocating the experimental tissue into a "normal" environment, all of these problems are avoided.

The transplantation of the fourth and ninth mammary glands of a mouse carrying the *FSP-cre* transgene into the interscapular region of wild-type mouse was used to determine the

practicality of this approach to address the issue. The *in situ* β-galactosidase assay confirmed that transplanted tissue can survive for up to sixteen weeks.

Discussion

From the very outset of this project, the goal was clear—to establish a way to genetically alter the stroma as to mimic the changes previously observed in the tumor microenvironment. One of those changes would be an increased deposition of ECM molecules such as is the case of carcinoma-associated fibroblasts (Sadlonova et al., 2005; Kunz-Schughart, 2002). Preliminary results of a study using the second generation *FSP-cre* transgene to delete the tumor suppressor *PTEN* show a marked increase in the deposition of collagen in the transplanted tissue (unpublished, Trimboli). The type of collagen and the affect that this has on the progression of the tumor has yet to be determined, but this finding is encouraging.

The implementation of a fibroblast-specific Cre recombinase could end up playing a vital role in future studies of tumor-stroma interactions; however, this approach to altering the stroma has its own limitations to be considered as well. The deletion of the target genes occurs very early in development and is unable to be used in studies requiring great control over the moment of initiation of an activated stroma. The next step is to establish a reliable inducible fibroblast-specific Cre recombinase system that would allow development to remain completely unaffected. A reverse transactivator would be a good choice because a response could be seen almost immediately; whereas, with a tet-off system, it is possible that tetracycline might remain in the tissue for an unknown period of time because of the surrounding adipose tissue. Another disadvantage of a tet-off system is that when dealing with deletion, if the mice fail to receive the appropriate amount of tetracycline at any moment, the gene is permanently lost from those cells that express the *FSP-cre* only briefly, as well as their descendent cells.

Overall, the value of the FSP-cre system is the ability to study the affects of an activated stroma *in vivo*. While cell lines like those of CAFs can be used to study communication between epithelial cells and fibroblasts *in vitro*, the ability to prove that *in vitro* results are actually the case *in vivo* will always be important. Even more important is the ability to transfer that knowledge into clinical practice. By elucidating the power of the stroma in the process of tumorigenesis and tumor progression, the prognosis and diagnosis for many patients could be made more precisely because the genetic alterations observed in the stroma which affect the rate of progression could be considered. With a less optimistic prognosis, a risky and more aggressive treatment might be used compared to a good prognosis. Understanding the role the tumor microenvironment plays in the initiation and growth of a tumor will have a direct affect on the treatment of the cancer patient.

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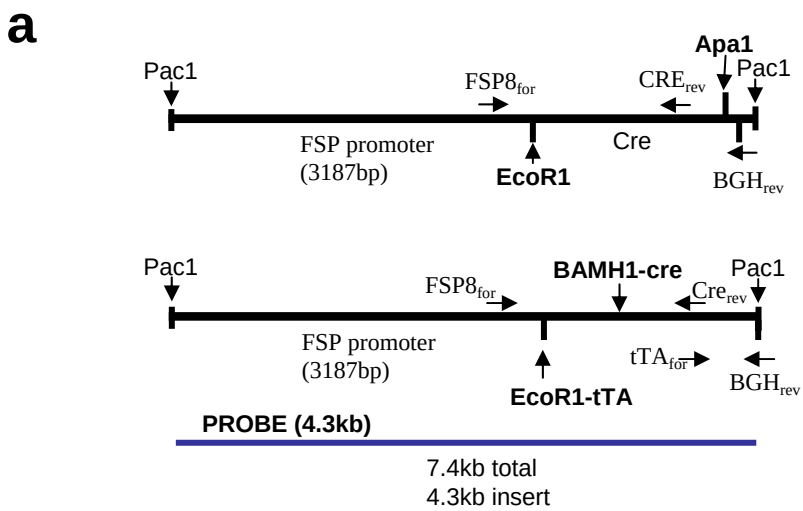


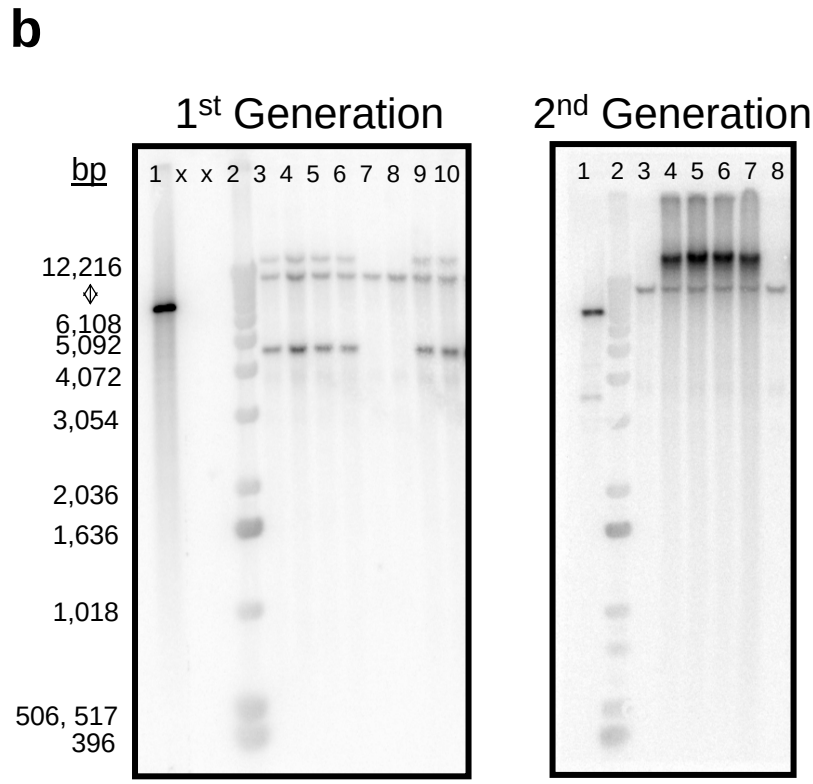


Fig. 1. Plasmid Illustration and Southern Analysis of the FSP-cre Founder Mice. **(a)** Schematic representation of the generated FSP-cre plasmid. The locations of the PCR primers used for genotyping (horizontal arrows) and restriction sites for (vertical arrows) are indicated. **(b)** Southern analysis of the FSP-cre insertion into the mouse genome. The endogenous *Fsp1* is present in all tail DNA samples (~10-11kb) Lane 1: FSP-cre plasmid. Lane 2: size marker. Lane 3+: tail DNA.

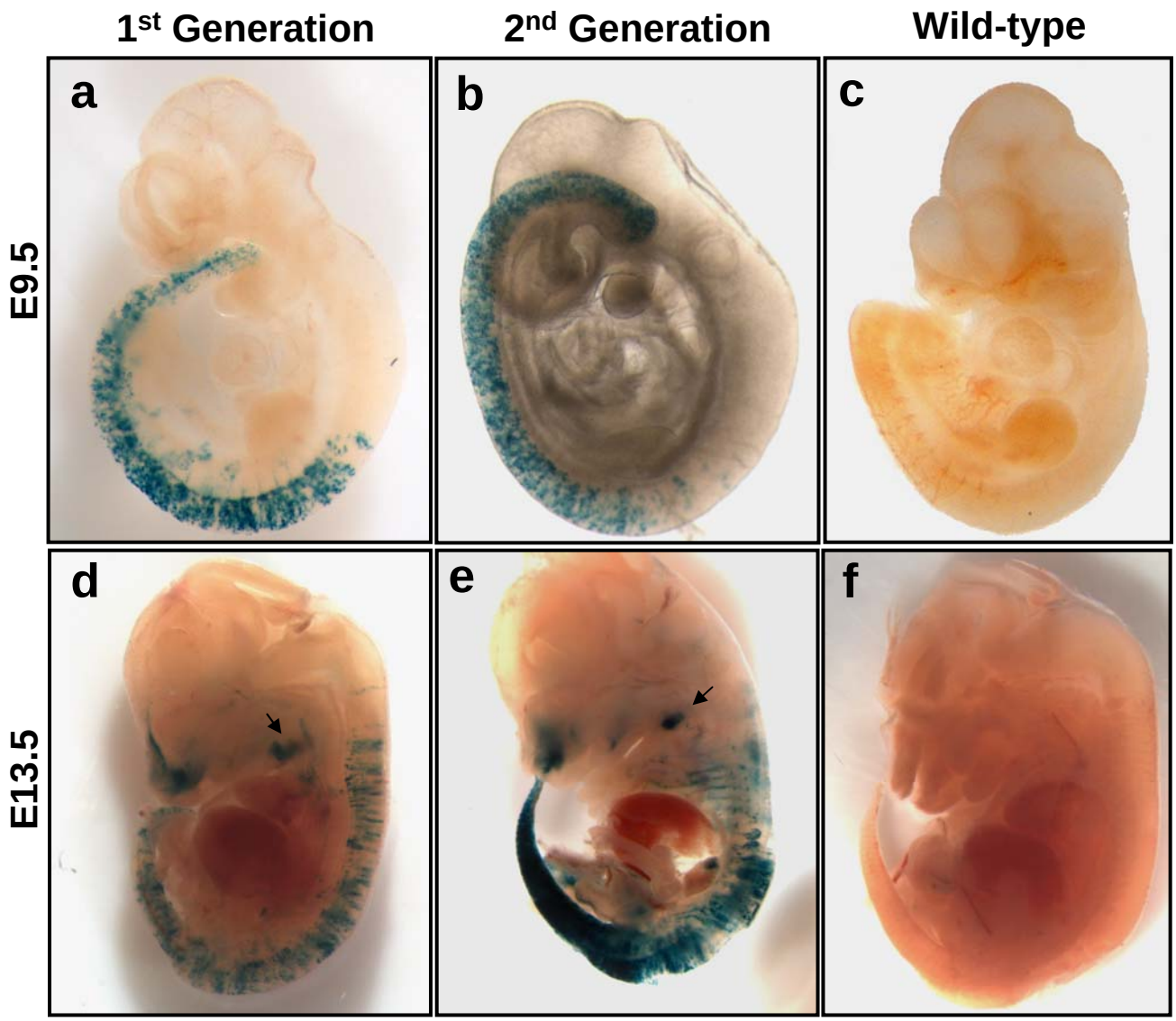


Fig. 2. Embryonic Expression Pattern Using FSP-*cre/Rosa*^{loxp} System. The utilization of X-gal staining to visualize FSP-cre expression in first generation FSP-cre (a,d), the second generation FSP-cre (b,e), and the wild-type (c,f) embryos at E9.5 (a-c) and E13.5 (d-f). The developing thymus in the E13.5 embryos is indicated by an arrow (d,e).

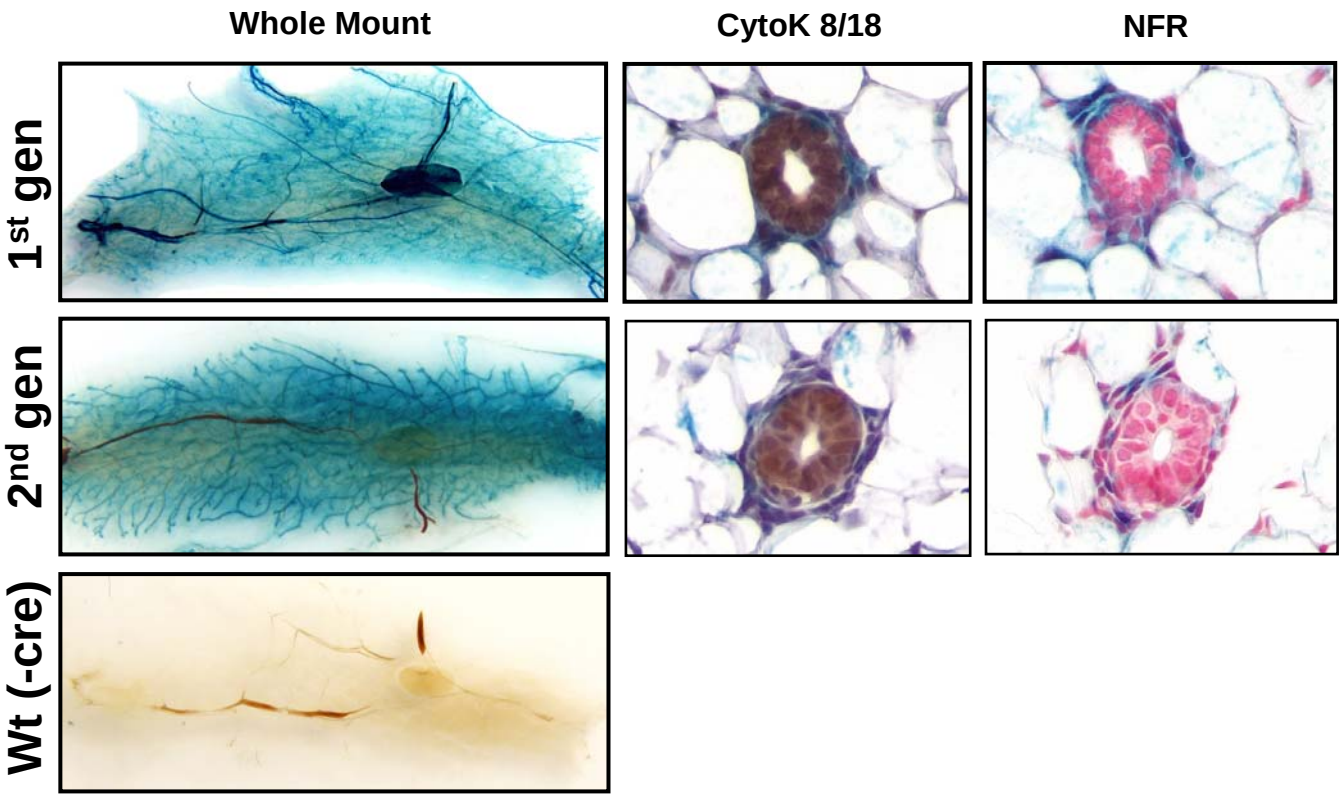
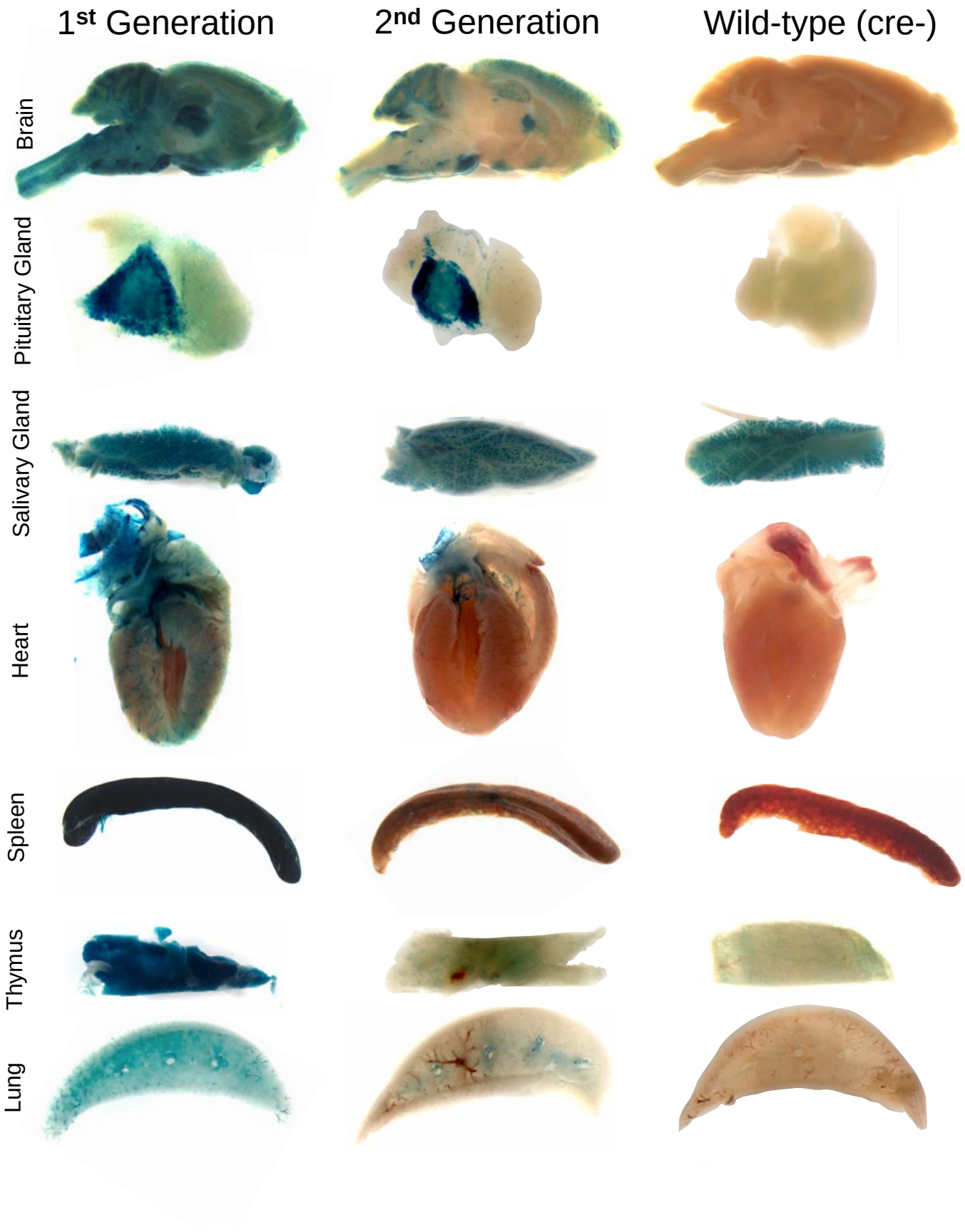
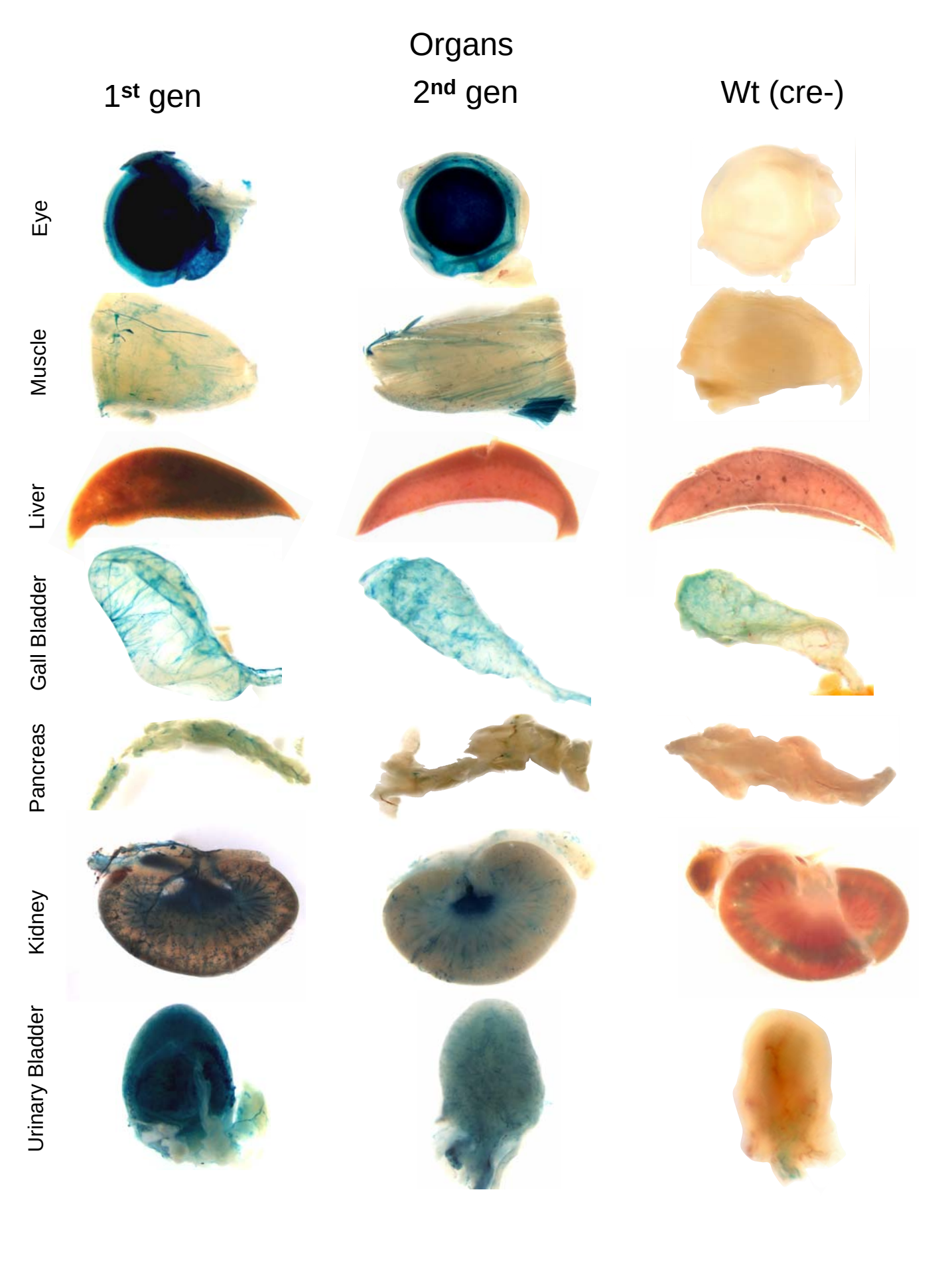


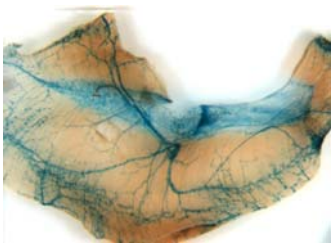
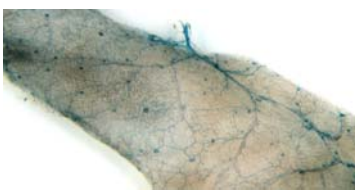
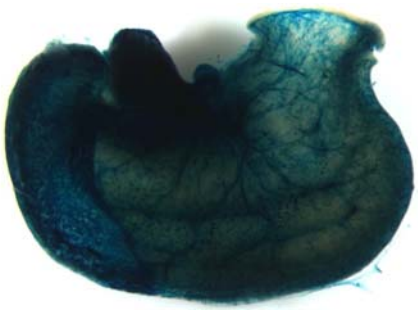
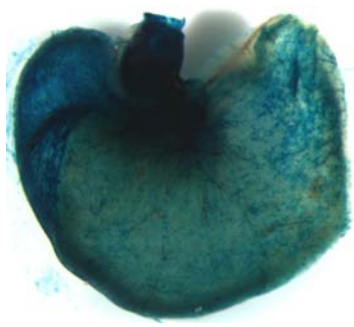
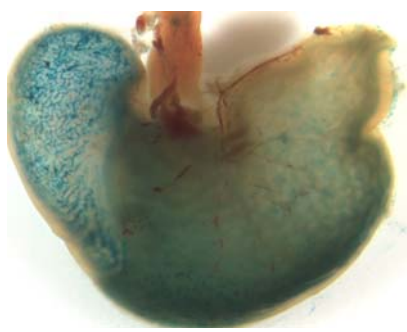
Fig. 3. Fibroblast Specificity in the Adult Mouse Mammary Gland. The visualization of the reporter gene *Rosa*^{loxp} to determine FSP-cre function and specificity in the first generation FSP-cre (a-c), the second generation FSP-cre (d-f), and the wild-type (g,h).

Fig. 4. *In situ* LacZ staining of the major organs.




Organs
1st gen
2nd gen
Wt (cre-)
Eye
Muscle
Liver
Gall Bladder
Pancreas
Kidney
Urinary Bladder

Organs

	1st gen	2nd gen	Wt (cre-)
Ovary and Uterus			
Peritoneal Membrane			
Diaphragm			
Intestine			
Stomach			

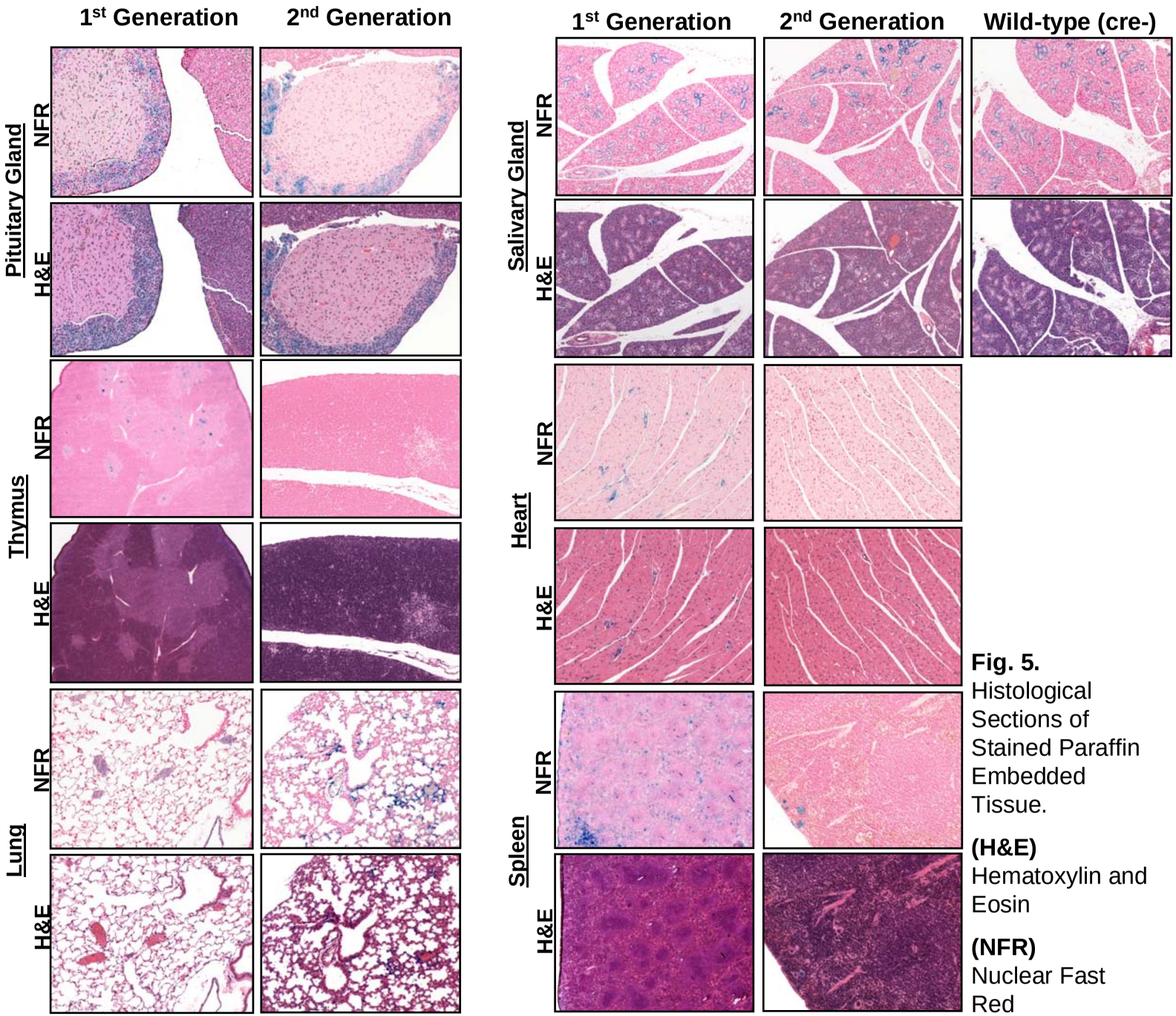
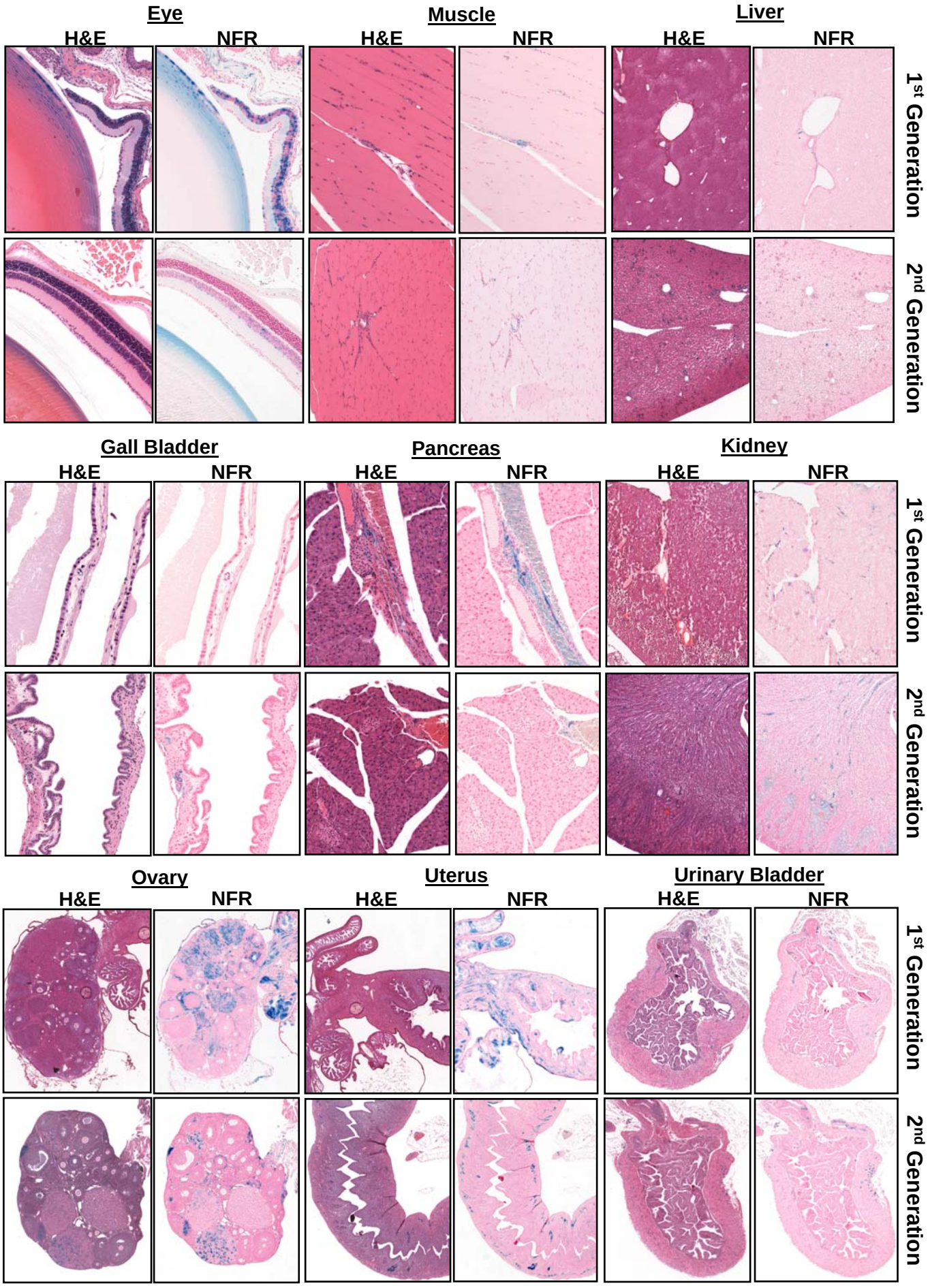
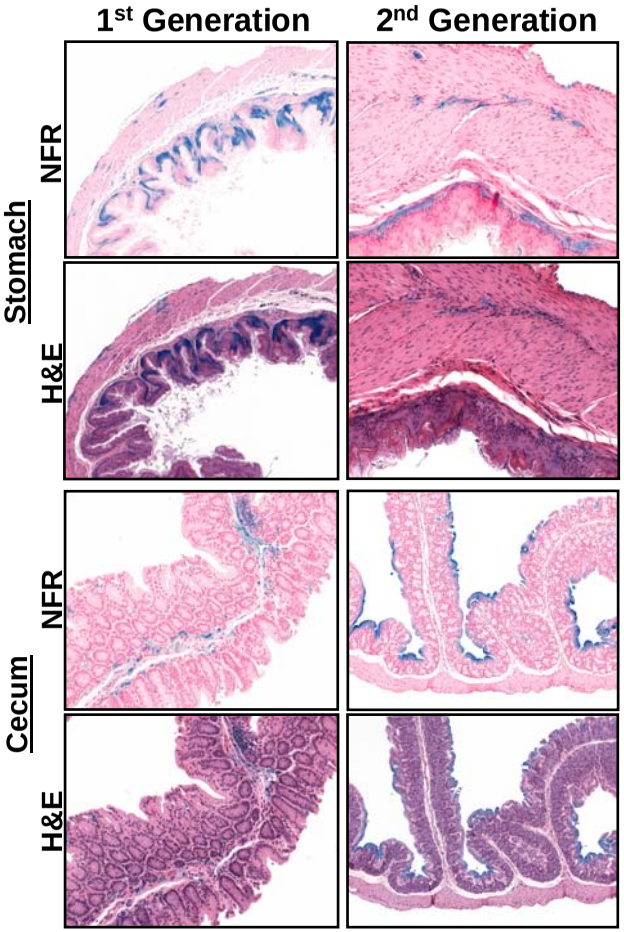


Fig. 5. Histological Sections of Stained Paraffin Embedded Tissue.

(H&E) Hematoxylin and Eosin

(NFR) Nuclear Fast Red


Eye
Muscle
Liver
H&E
NFR
H&E
NFR
H&E
NFR
1st Generation
2nd Generation
Gall Bladder
Pancreas
Kidney
H&E
NFR
H&E
NFR
H&E
NFR
1st Generation
2nd Generation
Ovary
Uterus
Urinary Bladder
H&E
NFR
H&E
NFR
H&E
NFR
1st Generation
2nd Generation


1st Generation
2nd Generation
Stomach
NFR
H&E
Cecum
NFR
H&E

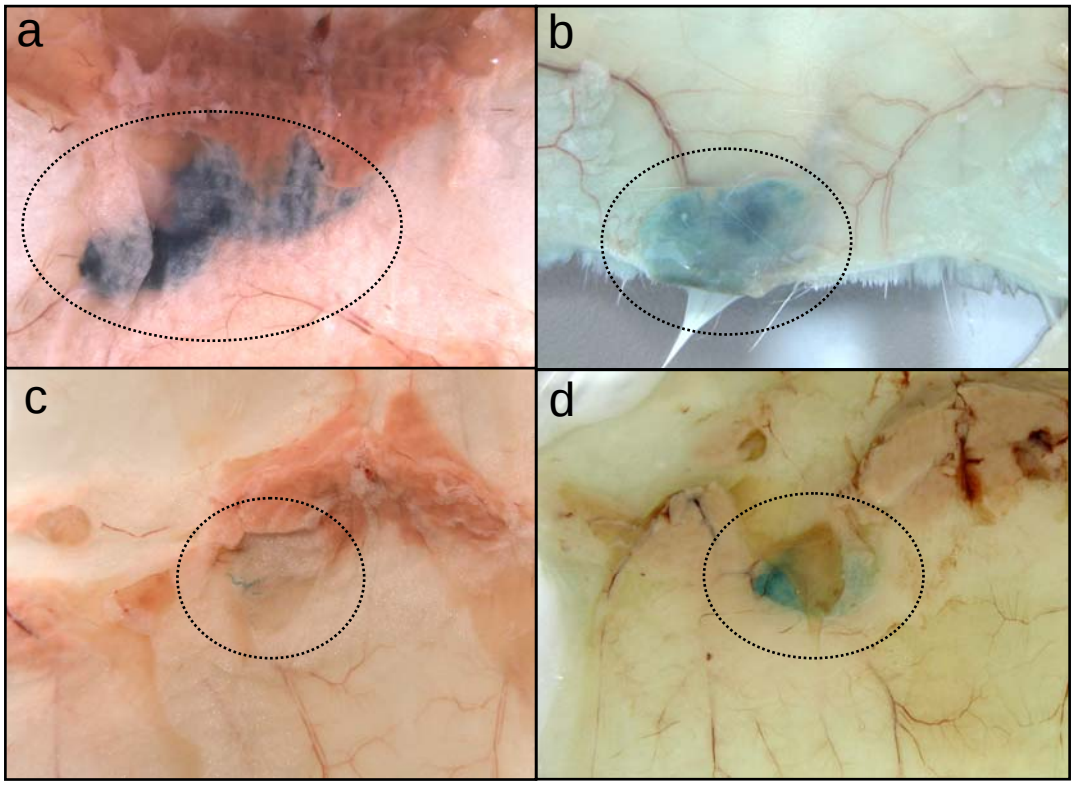


Fig. 6. Length of survival of stroma after transplantation. FSP-cre/Rosalox labeled 10th generation FVB/N mouse mammary gland tissue was transplanted to a wild-type 10th generation FVB/N host. Transplanted tissue is removed after **(a)** 2 weeks, **(b)** 4 weeks, **(c)** 8 weeks, and **(d)** 16 weeks. LacZ staining confirms the survival of the transplanted stroma for the designated period of time.

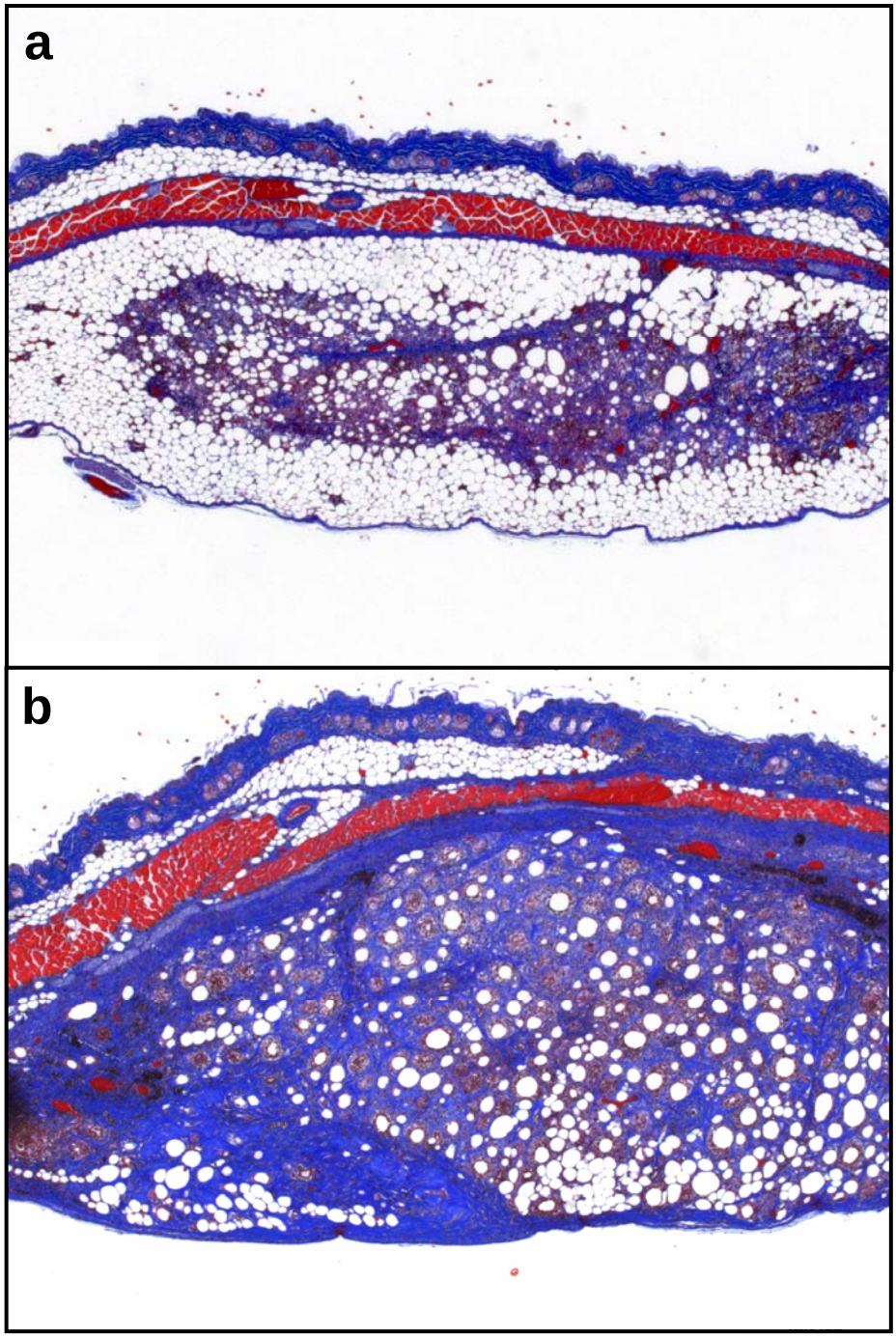


Fig. 7. Mason's trichrome staining of transplanted mammary glands 7 weeks after transplantation. The blue-staining material is collagen. **(a)** MMTV-c-neu without fibroblast-specific deletion of PTEN. **(b)** MMTV-c-neu with fibroblast-specific deletion of PTEN.

Table 1. Location of Positive LacZ Staining

Organ	Site of Positive LacZ Staining	Organ	Site of Positive LacZ Staining
Brain	1st gen: •Majority of brain 2nd gen: •Pons, thalamus, cerebellum, cerebrum, median eminence, area postrema	Liver	1st gen: •Around portal triads 2nd gen: •The positive staining is at a site of extramedullary hematopoiesis
Lung	1st gen: •Vasculature 2nd gen: •Near bronchial tubes	Gall Bladder	1st gen: •Vasculature 2nd gen: •Vasculature
Thymus	1st gen: •Spotted staining in medulla 2nd gen: •None	Pancreas	1st gen: •Vasculature 2nd gen: •Vasculature
Pituitary Gland	1st gen: •Pars intermedia 2nd gen: •Pars intermedia	Kidney	1st gen: •Vasculature •Collecting ducts 2nd gen: •Proximal tubular epithelium •Interlobar vasculature
Spleen	1st gen: •Multiple cell types throughout 2nd gen: •Sparse expression in cells of undetermined lineage	Ovary	1st gen: •Cells of the copora lutea •Theca interna around antral follicles 2nd gen: •Cells of the copora lutea •Theca interna antral follicles
Heart	1st gen: •Vasculature 2nd gen: •None	Uterus	1st gen: •Throughout endometrium & myometriium 2nd gen: •Uterine glands
Salivary Gland	1st gen: •Epithelial ducts (background) 2nd gen: •Epithelial ducts (background)	Urinary Bladder	1st gen: •Vasculature within the lamina propria and surrounding muscle 2nd gen: •Vasculature within the lamina propria and surrounding muscle
Eye	1st gen: •Inner nuclear layer of retina •Spotted exp. in ganglion •Epithelia surrounding lens 2nd gen: •Inner nuclear layer of retina •Epithelia surrounding lens	Cecum	1st gen: •Lamina propria portion of mucosa 2nd gen: •The surface of epithelium; most likely, remains from material being excreted.
Muscle	1st gen: •Vasculature 2nd gen: •Vasculature	Stomach	1st gen: •Epithelium of forestomach 2nd gen: •Submucosa and muscular tunic